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The Search for Scrapie Agent Nucleic Acid

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INTRODUCTION

The most fascinating and controversial data in the scrapie field are the numerous research papers suggesting that nucleic acid may not be responsible for scrapie infection. In one sense, owing to the ability of the agent to replicate and mutate, it would seem unreasonable for nucleic acid not to be involved. The bulk of scientific evidence to date would, however, argue that a modified host glycoprotein is essential for scrapie agent replication and that a conventional viral nucleic acid is not required for infection. In this article, we review the evidence supporting this protein replication hypothesis and describe the attempts to identify putative nucleic acids responsible for the scrapie infection.

DISEASE DESCRIPTIONS

Scrapie has been recognized as a slowly progressive neurologic disease of sheep and goats for 250 years. In 1936, Cuille and Chelle (14) showed that the disease was transmissible and confirmed that it had very long incubation periods of months or even years. This experimental observation, together with observations on sheep diseases in Iceland, were instrumental in formulating Sigurdsson's concept of slow infection (61).

Kuru, a disease of natives living in the highlands of Papua New Guinea, was shown in 1959 to be similar to scrapie (27) and was later transmitted to chimpanzees (26). Creutzfeldt-Jakob disease and the Gerstmann-Straussler syndrome are other transmissible presenile dementias of humans that, on the basis of clinicopathologic features and physiochemical studies of their transmissible neuropathogens, are indistinguishable from scrapie.

Another scrapielike disease, transmissible mink encephalopathy, was first recognized in 1965 (29) and was initially believed to be caused by feeding ranch-raised mink with scrapie-infected sheep. Epidemiologic studies on a new incidence of transmissible mink encephalopathy (43), together with the failure to demonstrate that scrapie strains tested in mink behave like agents of transmissible mink encephalopathy (42), suggest, however, that infected cattle may be a possible source of infection for this disease.

Chronic wasting disease of captive mule deer and elk was recognized when the brains of several animals with progressive debilitating neurologic illnesses were found to have typical scrapielike lesions of spongiform degeneration (68, 69). It is not known whether these diseases represent natural infection of these species or whether the animals are infected after confinement. Recent reports of scrapielike diseases of wild ruminants in zoological parks in Great Britain (32) suggest that infection, possibly from contaminated feed, may occur after capture.

The most significant new scrapielike disease of animals is bovine spongiform encephalopathy, reported in Great Britain in 1987 (65). The first affected cattle were observed in 1985, with the incidence gradually increasing to 1,400 cases per month. Studies on the epidemiology of bovine spongiform encephalopathy, with the aid of computer modeling, indicate that exposure was via a feed ingredient and began in 1982 with a 3- to 8-year incubation period. Assuming no cattle-to-cattle transmission, bovine spongiform encephalopathy is projected to continue at its present incidence until 1992 and then decline to zero over a 2- to 3-year period.

VIROLOGIC STUDIES ON THE ETIOLOGIC AGENT

In spite of intense research efforts, the cause of scrapie remains obscure. There is no pathogen-specific inflammatory response associated with the disorder. Viral particles associated with the disease have not been identified. Although the agent is filterable, its precise size has been the subject of much controversy. Some estimates of the size range from 4S to 10,000S (39, 51, 53), whereas other studies have indicated that the agent is more viruslike in size (21, 63). The agent is sensitive to proteinase K digestion (55), indicating the necessity of a protein component. The requirement for a nucleic acid component has not been established by physical methods. UV irradiation studies (described below) indicate that if nucleic acid is responsible for the disease, it is either very small or unusually well protected.

PRION PROTEIN: SUSCEPTIBILITY FACTOR OR TRANSMISSIBLE AGENT?

Most of the scrapie research over the past 10 years has been involved with the characterization of a host-encoded sialoglycoprotein. This protein has been shown to have a strong influence upon the scrapie incubation period and is believed by some investigators to be a necessary component of the infectious agent.

The discovery of this protein resulted from studies characterizing abnormal fibrils associated with scrapie-infected

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brain tissue. Merz et al. (45) were the first to identify these structures and referred to them as scrapie-associated fibrils (SAF). Two groups found that these abnormal structures were composed of a single glycosylated protein, of 27 to 30 kilodaltons if proteinase K was included in the extraction and 33 to 35 kilodaltons in its native form. One group referred to this molecule as the prion (for proteinaceous infectious particles) protein or PrP (50), whereas the second group, realizing that they had purified SAF, referred to the glycoprotein as SAF protein (20). These purified samples of SAF were found to be highly infectious and resistant to proteinase K treatment. The SAF (prion) protein is host encoded (5, 13, 30, 48) and is not protease resistant in uninfected animals. In addition to partial resistance to protease treatment, the infectious agent in these preparations is resistant to micrococcal nuclease, Zn^{2+} hydrolysis, and DNase I digestion (49).

The nomenclature of this glycoprotein is controversial and at times confusing. It is termed by some investigators SAF protein and by others the prion protein (PrP). The mature form has been variously referred to as Gp34 and PrP33-35, whereas the protease-resistant polypeptide has been termed PrP27-30. Since some protease-sensitive isoforms can have molecular masses of 30 kilodaltons or less, size designations for these proteins can be misleading. Following the nomenclature of Caughey et al. (10), we will use the term PrP-sens to indicate the protease-sensitive normal cell analog of PrP and PrP-res for the protease-resistant isoforms observed in scrapie-infected tissue.

Investigators studying purified SAF- and PrP-enriched preparations have reached contradictory conclusions on the importance of PrP-res. Some maintain that PrP-res itself is the infectious agent. Evidence supporting this theory includes the following: (i) PrP-res is the most abundant macromolecule in purified preparations; (ii) procedures that denature, hydrolyze, or modify the glycoprotein also diminish the titer; (iii) the PrP gene is linked to a gene controlling the incubation time of scrapie (*Sinc* gene); and (iv) mice with short and long incubation periods synthesize different PrPs (44, 50, 52, 54).

Other investigators have presented evidence that PrP-res is not essential for infectivity and have concluded that some other factor in the preparations is responsible for infectivity (2, 41, 46, 56, 62, 63). Evidence contradicting the prion hypothesis includes the following: (i) PrP can be separated from infectivity (2, 63); (ii) molecularly cloned PrP is not infectious (11); and (iii) deglycosylation of PrP does not affect infectivity (62).

The inability to identify differences in the primary structure of PrP has led some researchers to postulate that a posttranslational modification of the protein is responsible for the formation of the abnormal amyloidlike fibrils which somehow acquire the ability to transmit the disease (5, 64). The variability in protease sensitivity of the protein in infected and uninfected tissue may represent evidence for possible scrapie-specific posttranslational modifications or may simply reflect secondary changes in the protein owing to the disease process. The predominant association of PrP with pathologically damaged areas of the brain and the finding that its presence predates the appearance of structural lesions by several weeks (9) suggest that the accumulation of the abnormal fibrils may represent the primary injury to the cell.

The strong influence of the PrP gene on the incubation period of scrapie is well established. It appears to be identical to the mouse scrapie incubation (*Sinc*) gene characterized by classical genetics more than 20 years ago (16). The *Sinc* gene has been shown to have a significant effect upon the incubation period of a scrapie infection. By using the ME7 strain of the scrapie agent, two nondominant alleles, s7 and p7, were identified. Mice homozygous for the s7 allele had a much shorter incubation period than did those homozygous for the p7 allele. The *Sinc* gene has since been found to influence the incubation time in every known strain of mouse scrapie (18).

Almost 20 years after the demonstration of the existence of the *Sinc* gene by Dickinson et al. (16), Westaway et al. (66) were able to correlate the PrP primary sequence with the *Sinc* alleles for short and long incubation periods. Codon 108 of the prion gene was found to encode leucine in mice with short incubation periods while encoding phenylalanine in mice with long incubation periods.

Two recent studies provide perhaps the most compelling evidence of the involvement of the prion protein in determination of the incubation period of a scrapie infection. The first study involved a detailed analysis of the prion protein gene of individuals with Gerstmann-Straussler syndrome. This disease is a very rare transmissible human neurodegenerative disorder. It is usually familial and follows an autosomal dominant pattern of inheritance. Several investigators have correlated PrP gene variability with the disorder (22, 31). The second study took advantage of the species barrier effect to further delineate the role of the PrP gene in a scrapie infection. It is well established that a given strain of the scrapie agent will react differently in different organisms. For example, some mouse forms of the agent will not infect hamsters, and the 263K hamster agent is not pathogenic for mice. To test the hypothesis that the PrP gene may be involved in the species barrier effect, Scott et al. (60) produced transgenic mice containing the hamster form of the PrP gene and flanking regions. Inoculation of the hamster agent into the transgenic mice produced a scrapie infection, whereas inoculation into nontransgenic controls did not. Although approximately 30 kilobases of flanking-region genomic DNA was also included in the experiment (and therefore cannot be ruled out as having produced the effect), this experiment indicates that it is likely that the PrP gene controls host specificity for the scrapie agent.

Therefore, there would appear to be little doubt about the ability of the PrP gene to affect the incubation period in a scrapie infection. What is more controversial is whether a modified form of the protein is part of the infectious agent and whether if nucleic acid plays a role in the disease.

IS NUCLEIC ACID INVOLVED?

Central to biological dogma is the supposition that nucleic acids are the heritable material. In the field of scrapie research, this is, however, an issue of considerable controversy. Some researchers appear convinced that a modified form of PrP is the infectious agent. Their investigations therefore emphasize the identification of a posttranslational modification of PrP, which somehow induces disease and mimics the observation of self replication. Their nucleic acid studies are consequently limited to attempts to demonstrate that there is no nucleic acid basis for a scrapie infection. Other investigators suspect a requirement for an essential nucleic acid, either a yet undiscovered virus (63), an unusually structured nucleic acid, or a nucleic acid with significant sequence similarity to the host genome (1, 2).

It is well documented that the infectious agent plays a separate and distinct role from the PrP gene in determining

the nature of a scrapie infection. More than 15 different strains of the scrapie agent have been identified and characterized (8, 16, 17, 25). The majority of the scrapie strains produce incubation patterns similar to the ME7 strain, causing more prolonged incubation times in mice homozygous for the p7 allele than in those homozygous for the s7 allele. A few strains of the agent have, however, been documented to produce the opposite pattern (with p7 homozygous mice having shorter incubation times than s7 homozygous mice [15, 17, 18]). Further evidence that the scrapie agent is independent of the host genome includes mutation of the agent (8, 35), competition between strains of the agent (19, 36), and interspecies transmission (12, 28). The most obvious candidate for such a host-independent factor is nucleic acid.

Recent studies have identified nucleic acids associated with highly purified, highly infectious samples. Sklaviadis et al. (63) fractionated the Creutzfeldt-Jakob disease agent by using velocity sedimentation and isopycnic sucrose gradients. They found nucleic acid-protein complexes that comigrated with infectivity. Although PrP was also found associated with infectivity, the authors were able to dissociate the majority of the protein from infectivity. It was estimated that more than 95% of the nucleic acid detected was RNA. Two other studies have demonstrated the presence of nucleic acid in infectious PrP-res-enriched preparations that had been treated with proteinase K, Zn^{2+} hydrolyzed, and digested with both DNase I and micrococcal nuclease. Oesch et al. (47) identified host repetitive DNA sequences in the preparation. In the second study, relatively large amounts of mitochondrial DNA were found (1). These two studies argue that nucleic acids in the PrP-res-enriched preparations must be present in a highly protected form.

Agent inactivation studies with UV irradiation as well as ionizing radiation made it clear that if a nucleic acid was responsible for the scrapie infection, it was, at the very least, an unusual one. Studies by Alper et al. (3, 4) and Latarjet et al. (37) found the agent to be exceedingly resistant to UV irradiation, much more so than conventional viruses. As noted by many researchers (6), such an interpretation is open to criticism, since crude brain homogenates were used in the experiments. More recent studies have, however, produced similar results. Bellinger-Kawahara et al. (6), analyzing more purified fractions, have estimated that only a single-stranded nucleic acid of 5 nucleotides or a double-stranded nucleic acid of 25 nucleotides could survive the irradiation treatment. This study, however, is also subject to the same criticisms. Clearly, if nucleic acids are present in the preparation, their resistance to nucleases would argue that they are well protected. Therefore, although UV irradiation data certainly suggest that the putative scrapie nucleic acid is unusually well protected or may be quite small, it is premature to dismiss its presence altogether.

Similarly, ionizing radiation inactivation studies of the scrapie agent have been subject to considerable variability of interpretation. Some researchers have argued that the data support their contention that nucleic acid is not involved in scrapie infectivity (7). Rohwer (57, 58), comparing the scrapie inactivation rate constant with inactivation rate constants of viruses of known sizes, concluded that the scrapie genome responded in a manner consistent with that expected for small viruses.

SEARCH FOR SCRAPIE AGENT NUCLEIC ACID

A number of studies have used recombinant DNA methodologies to screen for nucleic acids unique to the scrapie infection. Recombinant libraries of nucleic acids isolated from scrapie-infected brain tissue have been screened by differential (plus-minus) hybridization (67). A 3.7-kilobase RNA, encoding glial fibrillary acidic protein, was identified as being preferentially expressed in scrapie-infected tissue. The more sensitive subtraction hybridization technique was also used to search for a nucleic acid unique to scrapie-infected tissue. These studies identified RNAs for the following proteins preferentially expressed in scrapie-infected brain tissue: glial fibrillary acidic protein, metallothionein II, B chain of α-crystallin, sulfated glycoprotein 2, and transferrin (23, 24). The increased abundance of these mRNAs during infection is believed to be the result of pathologic changes occurring in response to the infection and are not the primary cause of infection. The increased expression of glial fibrillary acidic protein has been shown by Mackenzie (38) to result from the prominent gliosis accompanying the disease. Similarly, sulfated glycoprotein 2 and transferrin, two transport molecules, may also be required to support the astrogliosis (23). It is speculated that expression of metallothionein II and the B chain of α-crystallin is increased as a result of stress (24).

Therefore, scrapie-specific nucleic acids have, to date, not been identified by differential hybridization methodologies. Although subtraction hybridization is the most sensitive method available for identifying nucleic acids unique to a given RNA population, scrapie-specific nucleic acids could be present that are not detected by the technique. For example, it has been estimated that the limit of detection for subtraction hybridization is 0.01% of the mRNA (59). Scrapie-specific RNAs present in very low abundance would therefore not be detected. In addition, the subtraction procedure will identify only those recombinants in one population that contain few similarities to the other nucleic acid population. If a scrapie-specific nucleic acid contains significant sequence similarity to nucleic acids in the uninfected population, it will not be identified. The construction of the cDNA library also eliminates certain RNA molecules from analysis. For example, cDNAs smaller than 150 nucleotides were excluded from the libraries (24). Finally, since only unique or preferentially expressed RNAs are detected by the subtraction method, a scrapie-specific DNA would not have been identified in these experiments. Therefore, if there is a nucleic acid involved in scrapie infection, it would appear to be either a unique DNA molecule, a very rare or very small RNA, or an RNA or DNA species having significant sequence similarity to nucleic acids present in uninfected tissue.

Our laboratory has recently demonstrated the presence of mitochondrial DNA in infectious nuclease-treated preparations enriched for PrP-res (1). An earlier study had suggested to us a possible mitochondrial involvement in the disorder (2). Mitochondria purified from scrapie-infected hamster brains were found to contain high infectivity. Removal of the mitochondrial outer membrane had no effect upon infectivity (2). If mitochondria are involved in a scrapie infection, one would expect mitochondrial nucleic acids to be present in the infectious PrP-res-enriched preparations. Analysis of these preparations demonstrated that a component of the mitochondrial genome, the small single-stranded D-loop fragment, was present in significant amounts (1). The D-loop fragment binds to the region of the mitochondrial genome that is involved in DNA replication and transcription. We are currently exploring the possibility that abnormal D-loop fragments are the basis for scrapie infection. We are hypothesizing that such nucleic acids would produce aberrantly

functioning mitochondria, which would be detrimental to the cell. A protein component (possibly PrP-res) could be required for protection or for cellular integration of the nucleic acid. This theory supports the virino hypothesis (19, 33, 34), which suggests that the scrapie agent consists of a host-encoded protein which provides protection for a nucleic acid that is responsible for the infection.

Other laboratories are also searching for scrapie-specific nucleic acids. Oesch et al. are in the process of analyzing recombinant DNAs synthesized from highly purified preparations enriched for PrP-res (47). Manuelidis et al. are basing their efforts on their data demonstrating that infectivity copurifies with nucleic acid complexes enriched for RNA and hypothesize a retroviral basis for the disease (40).

CONCLUSIONS

To date, scrapie research has produced substantial information on the effect of the host genome on the incubation period of scrapie. The scrapie incubation gene (*Sinc*) appears to be identical to the gene encoding PrP. Variability in the *Sinc* gene has been shown to have a significant effect on scrapie incubation time in mice. It plays a large role in the species barrier effect, and it has been correlated with susceptibility to Gerstmann-Straussler syndrome. It is also argued by some researchers that a modified form of the *Sinc* gene product is responsible for the scrapie infection. If this is true, the *Sinc* gene product may play a dual role in a scrapie infection, (i) affecting the incubation period of infection and (ii) being a component of the infectious agent.

Although an enormous amount of data has been accumulated documenting the influence of the *Sinc* gene (PrP gene) in scrapie infection, little has been learned about the agent itself. If nucleic acid is involved in infection, researchers have had little success in identifying it. This lack of success could be the result of the nucleic acids being unusually structured, exceedingly small, or low in abundance or containing significant sequence similarity to the host genome. Experimental approaches will have to address these considerations. The search continues.

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